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C3d positive donor-specific antibodies have a role in pre-transplant risk stratification of crossmatch positive HLA-incompatible renal transplantation: United Kingdom multicentre study.

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Abstract

Background: Anti-HLA antibody characteristics aid to risk-stratify patients and improve long-term renal graft outcomes. Complement activation by donor-specific antibody (DSA) is an important characteristic that may determine renal allograft outcome. There is heterogeneity in graft outcomes within the moderate to high immunological risk cases (crossmatch positive). We explored the role of C3d-positive DSAs in sub stratification of crossmatch positive cases and relate to the graft outcomes.

Methods: We investigated 139 crossmatch positive living donor renal transplant recipients from four transplant centres in the United Kingdom. C3d assay was performed on serum samples obtained at pre-treatment (pre-desensitisation) and day 14 post-transplant.

Results: C3d-positive DSAs were found in 52 (37%) patients at pre-treatment and in 37 (27%) patients at day 14 post-transplant. Median follow-up of patients was 48 months (IQR 20.47–77.57). In the multivariable analysis, pre-treatment C3d-positive DSA was independently associated with reduced overall graft survival, the hazard ratio of 3.29 (95% CI 1.37-7.86). The relative risk of death-censored five-year graft failure was 2.83 (95% CI 1.56 – 5.13). Patients with both pre-treatment and day 14 C3d-positive DSAs had the worst five-year graft survival at 45.5% compared to 87.2% in both pre-treatment and day 14 C3d-negative DSA patients with the relative risk of death censored five-year graft failure was 4.26 (95% CI 1.79, 10.09).

Conclusions: In this multicentre study, we have demonstrated for the first time the utility of C3d analysis as a distinctive biomarker to sub-stratify the risk of poor graft outcome in crossmatch positive living donor renal transplantation.

Abbreviations:

HLA, Human Leucocyte Antigen

ABMR, antibody mediated rejection

ATCR, acute T cell rejection

CDC, complement dependent cytotoxicity crossmatch

cPRA, Calculated Panel Reactive Antibodies

DSA, donor HLA specific antibodies

FC, flow cytometry crossmatch

IQR, Inter-Quartile Range

MFI, mean fluorescence intensity

rATG, rabbit Anti-thymocyte Globulin

SAB, Single Antigen Bead

Keywords

Donor Specific Antibodies
Risk stratification
C3d
Graft survival
Renal transplantation
Crossmatch

Introduction

It is known for the past five decades that the presence of complement activating anti-graft antibodies results in reduced graft survival and precludes transplantation (1). Early attempts at renal transplantation across antibody barriers have improved access to transplantation for highly sensitised patients (2-4). Refinement of desensitisation protocols has led to better survival for these patients compared to remaining on dialysis (5-8). However, even with significant progress in desensitisation immunotherapies, highly sensitised patients wait longer. Approximately thirty per cent of patients on the waiting list for deceased donor organs are highly sensitised and have calculated Panel Reactive Antibodies (cPRA)>20% (9). Recipients who have undergone HLA incompatible transplants are at higher risk for both acute antibody mediated rejection (ABMR) and shortened graft survival compared to those who have received compatible or ABO-incompatible transplants. Despite advances in immune monitoring technologies, uncertainties with pre-transplant risk stratification prevail. CDC positive crossmatch is associated with the highest risk of both an ABMR and graft failure. The risk is lesser if the DSA is detectable only by FC crossmatch and the risk falls further in crossmatch negative cases, where DSA is detected by single antigen bead (SAB) analysis (10-12). Graft failure is usually related to chronic antibody mediated injury(11, 13-15).

Numerous investigations have begun to establish the role of DSA in mediating renal allograft rejection and graft survival (16-18). We have previously shown that following HLA incompatible transplantation, anamnestic responses peak at around 14 days post-transplant and the risk of acute ABMR is higher with DSA MFI >7000 (19). Anti-HLA subclasses IgG1 and IgG3 strongly activate complement and have the potential to serve as predictors of rejection and allograft survival (20-22). The field of antibody testing has evolved, and it is possible to study the complement proteins and split products on solid-phase assays using isolated HLA proteins (23-25). The advancements in immune monitoring emphasise the need to investigate the role of DSA that activates the complement cascade in the highly

sensitised patients with positive crossmatch, both pre-treatment and following early transplantation. Awareness of the presence of complement activating DSA could potentially enable effective risk stratification of crossmatch positive patients as well as the prediction of ABMR. However, studies in standard or low immunological risk (crossmatch negative) patients, complement-fixing assays have shown conflicting results in risk stratification for ABMR and graft survival(24, 26-31). In this multicentre study, we investigated the role of pre-treatment and early post-transplant complement activating DSA (as measured by C3d assay) to aid in further risk stratification of moderate to high immunological risk (positive crossmatch) cases, for renal allograft survival.

Concise Materials and Methods

Study design

This analysis comprises a United Kingdom multicentre retrospective cohort study of patients who underwent HLA antibody incompatible renal transplants at four University Hospital centres: University Hospitals Coventry and Warwickshire (UHCW); Guy's Hospital, London; University Hospital of Wales (UHW, Cardiff), and Leeds Teaching Hospitals (LTH). Ethics committee approved the study (Ethics reference number is CREC-055/01/03 and 13/WM/0090). Clinical and research activities abide by the 2013 Declaration of Helsinki and the 2008 Declaration of Istanbul.

All of the following inclusion criteria were met: recipients of living donor HLA antibody incompatible transplants with pre-transplant plasmapheresis; CDC and/or FC crossmatch positive and DSA positive by SAB before desensitisation therapy

The exclusion criteria were: recipients with DSA detected by SAB alone (i.e. crossmatch negative) that did not require pre-transplant plasmapheresis; dual HLA and ABO-incompatible renal transplants; pediatric renal transplant recipients (age<18 years); and primary non-function of the graft.

One hundred and ninety-nine recipients received antibody incompatible renal transplants between 2005 and 2015 at the four transplant centres, of whom 139 met the inclusion criteria and comprised the final study cohort. We analysed pre-treatment (pre-transplant sample before plasmapheresis and/or IVIG) and day 14 samples (19, 32-34). Pre-treatment serum samples were available for 139 recipients, but post-transplant day 14 samples were only available for 111 recipients.

HLA typing and antibody testing.

Histocompatibility and immunogenetics laboratory performed the HLA typing and serological testing. HLA types were determined by DNA-based methods for HLA-A, B, C, DR, DPB and DQB alleles. CDC (without AHG enhancement) and FC assays were performed as published in our previous study (13). CDC crossmatch was performed only in three of the four centres. All the stored pre-treatment serum samples were tested for IgG antibodies at a final dilution of 1:5 as per the manufacturer guidelines (Lifecodes, Immucor, UK) and other published literature (29, 35). This technique reduced the commonly encountered problem of high dose hook effect (35). The detailed method is described in the supplemental material. Data acquired with Luminex version 2.3 were analysed with MatchIT software provided by the manufacturer.

C3d analysis

The C3d analysis was performed with the C3d assay kit (Lifecodes®, Immucor). Both pre-treatment and day 14 samples were tested for C3d deposition on the HLA single antigen beads as per manufacturer guidelines. C3d data acquired with Luminex version 2.3 were analysed with MatchIT software provided by the manufacturer.

Desensitisation protocol

Patients with positive CDC and/or positive FC crossmatch against their donors typically underwent between three to seven sessions of plasmapheresis with or without IVIG to render them CDC and/or FC crossmatch negative at the time of the transplant.

Desensitisation protocols, induction agent and maintenance immunosuppression followed at the four centres are summarised in Supplemental Table 2.

Rejection episodes were diagnosed with renal biopsy findings in line with contemporary Banff criteria (36-38).In eight cases where the biopsy was precluded for reasons such as anticoagulation, patient refusal, the diagnosis of ABMR was based on clinical findings (drop in urine output with rising creatinine) and the lab data (rapidly rising DSA levels). ABMR episodes within the first-month post-transplant were considered as early ABMR. Rejection episodes were treated initially with intravenous methylprednisolone. Other treatment for ABMR included rATG (21 patients), plasmapheresis and IVIG (3 patients) and two recipients with refractory ABMR received rituximab and eculizumab.

Statistical analysis

Statistical analysis was performed using the Pearson Chi-square test for categorical variables. Normality of data was tested using the Shapiro Wilk test. Independent samples t-test and Mann Whitney U test was used for continuous data depending on the distribution of data. Multivariable analysis for medium-term survival outcome was carried out using Cox proportional hazard models. We chose three different models to determine the independent role of C3d DSA after adjusting for variables. IgG DSA variable was studied differently in the models (In model 1- continuous variable, models 2 and 3- as a categorical variable with different cut-offs of 8000 and 5000). The IgG DSA arbitrary cut-offs were based on association with FC or CDC reactivity at our centres. IgG DSA cut off ≥8000MFI is associated with higher positive predictive value for positive CDC crossmatch (39, 40)

Kaplan Meier survival analysis and adjusted death-censored graft survival analysis were carried out after adjusting for age, gender, duration on dialysis, mismatch, previous transplantation and ABMR. Groups were compared based on the Log-rank test. For clinical relevance, relative risks for death censored five-year graft failures were computed using an online calculator for effect size measures (41). The null hypothesis of no difference between

the groups of interest was tested at the 5% significance level. Calculations were performed using SPSS V24 (Chicago, IL).

Results

Characteristics of the study population

The characteristics of the 139 recipients are presented in Table 1. The median follow-up time was 48 months (IQR 20.47–77.57). Fifty-two (37%) pre-treatment and thirty-seven (27%) post-transplant cases had C3d-positive DSA. Characteristics that were associated with a higher proportion of C3d-positive DSAs included younger age, male gender, longer dialysis duration and patients with previous transplants. There were no significant differences between the groups with regards to immunosuppression at induction apart from a higher proportion of cases with C3d positive DSA receiving rATG, but overall numbers were low. Pre-treatment C3d-positive DSA was associated with higher IgG DSA MFI and was predominantly HLA class II antibodies. Thirty-five grafts failed over the entire period of whom pre-treatment C3d-positive DSA were present in twenty-two cases (Supplement Table 3). Six patients died with functioning grafts during the follow-up. None of these patients had pre-treatment C3d-positive DSA. Only one patient had day 14 C3d-positive DSA.

C3d analysis and renal allograft survival

Over the study period, thirty-five (25%) grafts were lost. Twenty-nine cases had renal biopsy features consistent with chronic antibody mediated rejection. Other causes included acute ABMR (one case); recurrence of glomerular disease (four cases), and sepsis (one case). Twenty-nine grafts failed at five years. The relative risk of death censored five-year graft failure in pre-treatment C3d-positive cases was 2.52 (95% CI 1.29 – 4.91). The difference in survival proportions for C3d-positive DSA group at five-years was -0.39 (95% CI -1, 0.686).

The association of pre-treatment C3d-positive DSA with renal allograft survival was investigated in a multivariable Cox proportional hazard analysis, using the three models, as

shown in Table 2. In Model 1 with IgG values taken as a continuous variable, the presence of pre-treatment C3d-positive DSA was associated with worse graft survival, hazard ratio 3.29 95%CI 1.37-7.86 (p=0.007) (Table 2a) (Figure 1).

In model 2, IgG DSA MFI was categorised into ≥8,000 and <8,000. Sixty-five patients had pre-treatment IgG DSA MFI >8,000, of whom forty-three were C3d-positive. Of seventy-four patients who had IgG DSA MFI levels <8000, only nine were C3d-positive. Pre-treatment C3d-positive DSA cases were associated with lower graft survival compared to C3d-negative DSA cases, hazard ratio 2.98 (95%CI 1.29 – 6.88) (p=0.011) (Table 2b).

In model 3, IgG DSA MFI was categorised into ≥ 5000 and <5000. Ninety-one patients had pre-treatment IgG DSA MFI >5000 of whom forty-eight were C3d positive, and forty-eight patients had an IgG DSA MFI <5000 of which four were C3d-positive. Pre-treatment C3d-positive DSA cases were associated with lower graft survival compared to C3d-negative DSA cases, Hazard ratio 3.31 95% CI 1.45 -7.58 (p = 0.004) (Table 2c).

In all the three models, pre-treatment C3d-positive DSAs were independently associated with poor renal allograft survival. IgG DSA were not significantly related to graft survival in any of these models. Previous kidney transplantation and ESRD duration were other independent significant factors.

Subgroup analysis

C3d-positive DSA and graft survival in flow crossmatch positive patients

Of 139 cases, twenty-six were CDC positive. Forty cases did not have CDC tested; hence we looked at seventy-three cases that were only FC positive as a subgroup to explore sub-stratification using C3d. Eleven grafts failed over the study period in this group. C3d-positive DSA present in seventeen cases.

The death-censored graft survival at five-years in C3d-positive DSA cases were lower at 64.5% compared to 90.7% in C3d-negative DSA cases. The relative risk of five-year graft

failure was 3.74 (95%CI 1.12, 12.49). The difference in survival proportions for C3d-positive DSA group at five-years was -0.25 (-0.53, 0.014).

In the multivariate cox proportional hazards analysis (model 1), C3d-positive DSA cases were associated with lower graft survival compared to C3d-negative DSA cases. The hazard ratio was 9.8 (95%CI 1.6, 59.4). (Figure 2).

Graft Survival according to pre-treatment C3d-positive DSA HLA class

Of 139 cases, IgG HLA class I DSAs were present in 111 cases, and IgG HLA class II DSAs were present in eighty-six cases. Sixty-one cases had both HLA classes I and II IgG DSAs. Fifty-two cases had either HLA class I, HLA class II or both HLA classes of pre-treatment C3d-positive DSAs. HLA class I C3d-positive DSA only were present in fifteen cases, HLA class II C3d-positive DSA only were present in twenty-nine cases and both HLA classes I and II DSA only were present in four cases. Of the fifteen HLA class, I C3d-positive DSA cases, and there were four graft failures over five years.

In Kaplan-Meier survival analysis, death censored graft survival at five-years in cases with pre-treatment HLA class I C3d-positive DSA were marginally lower at 73.3% compared to 75.5% in HLA class I C3d-negative DSA cases (Figure 3). The relative risk of death censored five-year graft failure was 1.06 (95%CI 0.33, 3.42).

In Kaplan-Meier survival analysis, the death censored graft survival probability at five-years in the class II C3d-positive DSA cases were 60.7% compared to 80.6% in class II C3d-negative DSA cases (Figure 4) with the relative risk of 2.02 (95% CI 1.04 – 3.94)

In Kaplan Meier survival analysis, the death censored graft survival probability at five-years with both class I and II C3d+DSA was lower at 50% compared to 80.7% in rest of the cases (Figure 5)with the relative risk of 2.05 (95%CI 0.73, 5.78).

Persistent C3d-positive DSAs and death censored graft survival analysis:

One hundred eleven cases with both pre-treatment and day 14 samples were studied. C3d status at pre-treatment and day 14 were as follows. Fifty-seven cases had C3d-negative DSA for both pre-treatment and on day 14 sample; twenty-four cases had both pre-treatment and day 14 C3d-positive DSA; Sixteen cases had pre-treatment C3d-positive DSA but negative on day 14, and fourteen cases had negative pre-treatment but C3d-positive DSA on day 14.

In the Kaplan Meier survival analysis, death censored five-year graft survival was worse in cases with both pre-treatment and day 14 C3d-positive DSAs (43.5%) compared to cases with both pre-treatment and day 14 C3d-negative DSAs (87.2%) (log-rank p = <0.001) (Supplement figure 1).

In the death censored five-year graft survival analysis, cases with pre-treatment and day 14 C3d-positive DSAs were associated with worse five-year graft survival of 45.5% compared to 87.2% in cases with pre-treatment and day14 C3d-negative DSAs (Figure 7). The relative risk of graft failure was 4.25 (95%CI 1.79, 10.09).

The relative risk of death censored five-year graft failure, according to C3d status, is presented in Figure 6. There were mainly four categories. In cases with pre-treatment C3d-positive DSA and day 14 C3d-negative DSA compared to cases with pre-treatment and day 14 C3d-negative DSA was 2.07 (95%CI 0.59, 7.28); The relative risk of five-year graft failure in cases with pre-treatment C3d-negative and day 14 C3d-positive DSA compared to cases with pre-treatment and day 14 C3d-negative DSA was 2.03 (95%CI 0.58, 7.12).

In a multivariable Cox proportional hazard analysis (model1), cases with pre-treatment and day 14 C3d-positive DSA were associated with lower renal allograft survival compared to pre-treatment and day 14 C3d-negative DSA cases. The hazard ratio was 4.56 (95%CI 1.46-14.39, p=0.009) (Table 3) Models 2 and 3 had similar results (data not included, available if necessary).

Discussion

This multicentre study illustrates the role of C3d assay in the sub-stratification of graft survival risk in crossmatch positive cases, for the first time. C3d assay was able to clearly define groups with better outcome among moderate to high immunological risk cases (Figure 6). The best graft survival is seen in patients with C3d-negative DSA at both pre-treatment and day 14 (Figure 7), which is comparable to five-year graft survivals in deceased donor transplants in the United Kingdom. However, marginally lower than standard living donor transplants (9, 42, 43) The most inferior graft survival was found for those with C3d-positive DSAs in both pre-treatment and day 14 sera. Thus, the lower risk cases can be predicted before the transplant. In contrast, the highest risk cases can be assessed if there is additional testing in the early post-transplant period, long before the actual time of failure, which indicates there is a potential for intervention in the early post-transplant period.

Previous studies have only explored the role of complement activation DSA assays in a predominantly lower immunological risk group (DSA alone positive with crossmatch negative cases). In a single centre study, of 68 highly sensitised patients (twenty-one CDC crossmatch positive patients) looked at the pre-treatment risk stratification using in-vitro C4d deposition on SAB. Presence of pre-transplant C4d-positive DSAs were associated with acute AMR. One, three, and five-years death censored graft survival was also significantly lower in the C4d-positive DSA patients compared to C4d-negative DSA patients (44). Similarly, other studies have shown that in crossmatch negative, sensitised patients with complement activating DSAs, as measured by C1q binding at the time of transplantation and/or post-transplantation are associated with poor renal allograft survival (26, 45, 46). A large study by Kamburova et al. concluded that the presence of pre-transplant C3d-positive DSAs were associated with reduced renal allograft survival but did not reach statistical significance (29). Possible reason could include the study cohort that comprised of crossmatch negative DSA positive cases (different from our study cohort). Another possible

explanation is consideration of only pre-transplant status of the DSAs. In other studies on paediatric renal transplant population, complement activating DSAs in the post-transplant period is a risk factor for graft failure (47, 48).

Positive complement activation assays of DSAs utilised at other time points (such as graft dysfunction or ABMR) following transplantation were also associated with inferior graft outcomes. Two comparable studies have indicated that testing for complement activating DSA (C1q binding and C3d activation assays) at the time of AMR predicts graft survival (24, 26). Similarly, a recently published study from the deteriorating kidney allograft function (DeKAF) investigators, in a cohort of standard risk renal transplants, the results of a C3d assay performed at the time of development of DSA and graft dysfunction, predicted a higher risk of graft failure in C3d-positive DSA group compared to C3d negative DSA group (28).

Thus, a common thread in these studies is that the combination of pre- and post- renal transplantation testing for complement activating DSA can be of predictive value, and their use, particularly in immunologically high-risk cases, is becoming compelling. Which measure of *in vivo* complement activating potential, C1q binding or C3d generation is superior, if at all, remains to be proven; the study by Kim et al. and Lee et al. (25, 49) suggests the latter. Each assay measure different DSA properties; C1q binding being dependant on Fc density on the antibody target, while C3d generation quantifies the full activation potential of the DSA (including Fc cross-linking). C3 activation is the pivotal reaction of the complement cascade, which leads to the production of inflammatory mediators and direct tissue damage. As such, the C3d assay would appear to be the more relevant measure of DSA pathogenicity, which is why we chose it for this study.

Also, we did not find a significant linear correlation between C3d and higher IgG MFIs as compared to other studies using C1q and IgG MFI (50, 51) and C3d and IgG MFI (39) (Supplement Figure 2). There remains to be proven what an MFI value means for complement assay (either C1q or C3d), as a higher MFI for complement split product may

not necessarily correlate in a linear fashion to the quantity similar to IgG MFI. Thus, in this study cohort, we have used only categorical/qualitative results.

It is established that complement activating antibodies, as detected by complement-dependent cytotoxicity crossmatch, are associated with a worse graft survival (1). However, the CDC assay is not always specific and can identify other, non-HLA complement activating antibodies (51, 52). The availability of viable donor cells still limits the CDC assay, and it is not always practical to perform this assay on multiple occasions in the pre-transplant and post-transplant periods. Correlation of complement activating DSA assays with CDC/FC crossmatches are of interest. In recently published studies, that included samples from single centre of this cohort, a positive C3d assay was shown to correlate with high specificity, and a positive predictive value with positive FC crossmatch. However, sensitivity and negative predictive values were low (39, 40). A negative C3d assay had a higher negative predictive value for CDC, and this could be a useful surrogate marker for risk stratification, as negative CDC crossmatch is generally required at the time of transplantation.

The flow cytometry cross matches, although more sensitive than CDC, has similar limitations to the CDC assay. Typically, a positive pre-transplantation crossmatch is discouraged due to the high risk of rejection (53) and reduced graft survival (10, 11). Subgroup analysis of FC crossmatch patients in this study showed pre-treatment C3d-positive DSAs were associated with lower graft survival (Figure 2). The findings are not entirely surprising as one of the earlier studies that looked at the utility of C4d-positive DSA in a cohort of highly sensitised patients, presence of pre-transplant C4d positive DSA in CDC crossmatch patients were associated with worse graft survival compared to C4d-negative cohort (54). The above findings demonstrate the potential utility of C3d testing in crossmatch positive patients.

In the subgroup analysis of graft survival based on HLA class I and class II; cases with both HLA classes C3d-positive DSAs, had lower graft survival as compared to other cases. Presence of only HLA class II C3d-positive DSAs reached statistical significance. Limited

number of studies have looked at the HLA class of complement activating DSAs and majority of these studies are limited by relatively low numbers of patients. In a recent study, denovo class II C3d-positive DSAs were associated with higher rejection episodes and significantly lower graft survival (25). A study that looked at the effect of C4d-positive DSA class on ABMR, found no significant difference (44). However, a previous study from the same group in found lower graft survival in the presence of HLA class I C4d-positive DSA but not with HLA class II C4d-positive DSA (54). Studies that have looked at HLA class difference of DSAs on allograft survival have not found statistical significance. However, recent studies have demonstrated lower graft survival with post-transplant denovo complement activating HLA class II DSAs. (26, 55, 56).

The strengths of our study include multicentre validation of the C3d assay in a large cohort of crossmatch positive patients. Also, patients received relatively homogenous maintenance immunosuppression. However, this is a retrospective study and post-transplant day 14 samples were not available for all 139 cases. Heterogeneous induction regimen may have affected the long-term outcomes, although there was no statistical significance between the groups (Supplement Table 4). It is also not possible to extrapolate optimal treatment strategies for early post-transplant rising DSA levels with no graft dysfunction, as protocol biopsies were not performed. Further mechanistic studies are required to explore the potential for therapeutic options in the early post-transplant period. One of the pathways studied includes, inhibiting the functions of active complement fragments such as C3d, which has a role in augmenting B cell-mediated alloimmunity. (57). Complement regulatory proteins, both membrane-bound and circulating, play a role in the susceptibility of microvascular endothelial cells to complement mediated damage. Augmenting the expression of CD55, CD46 or CD59 on endothelial cells may reduce the cytotoxicity of complement activating antibodies (58, 59). There are potential diagnostic and therapeutic implications for identifying critical pathological pathways, including complement

activation(60, 61). We speculate that rendering the C3d negative before a transplant can result in better graft survival.

In conclusion, C3d assay enabled differentiation of IgG antibodies with varying pathogenicity. Thus, it has a potential role as an additional biomarker in further risk assessment of transplant patients, especially in cases with preformed DSAs and positive crossmatch results against their potential donors. Further prospective controlled studies are required in high immunological risk patients to establish the role of this approach.

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Table 1
Baseline characteristics and outcomes

Characteristics (n)	All patients	Pre-treatment C3d positive	Pre-treatment C3d negative	*p*-value
Number of patients	139	52/139	87/139	
Age, mean (SD)	42 (11.38)	38 (9.7)	44 (11.8)	***0.003***
Gender, male, n (%)	50 (36)	25 (48)	25 (29)	***0.021***
Previous transplantation, n (%)	88 (63)	40 (77)	48 (55)	***0.01***
Dialysis years, median (IQR) mean rank	8 (3 - 16)	14.5 (8 - 18.75) 80.15	7 (3 -15) 63.93	***0.02***
Mismatch, (A, B and DR) median (IQR) mean rank	3 (2 - 4)	3 (2 - 4.75) 78.12	3 (2 - 4) 65.15	0.06
DR mismatch, median (IQR) mean rank	1 (1 - 1)	1 (1 - 1) 75.44	1 (1 - 1) 66.75	0.15
Basiliximab (%)	92 (66)	32 (62)	60 (69)	0.48
Alemtuzumab (%)	33 (24)	11 (21)	22 (25)	0.70
rATG (%)	14 (10)	9 (17)	5 (6)	0.06
Median highest IgG MFI, (IQR) Mean Rank	7192 (3713 – 12278	12679 (10003 - 16254) 100.92	4949 (2328 - 7912) 51.52	***<0.001***
DSA Class I only, n (%)	54 (39)	7 (13)	47 (54)	***<0.001***
DSA Class II only, n (%)	27 (19)	16 (31)	11 (13)	***0.014***
Class I+ II, n (%)	58 (42)	29 (56)	29(33)	***0.013***
Day 14 C3d+DSA, n (%)	37(34)	23(44)	14(16)	***<0.001***
Early ABMR, n (%)	49 (35)	21 (40)	28 (32)	0.36
ACR, n (%)	16 (11)	5 (10)	13 (15)	0.44
Mixed rejection, n (%)	10 (7)	3 (6)	7 (8)	0.74
Graft loss, n (%)	35(25)	22(42)	13(15)	***<0.001***

*Statistical tests used: Age-Independent samples t-test, Gender, previous transplants, Immunosuppression, DSA class, ABMR, ACR and Graft loss-Pearson Chi-square test. Dialysis years, mismatch, IgG DSA MFI- Mann Whitney U test.

Table 2

Table 2a Model 1				**Table 2b Model 2**				**Table 2c Model 3**			
Multivariable Cox proportional hazard model. Pre-treatment C3d-positive DSA was predictive of poor graft survival.				Multivariable Cox proportional hazard model. Pre-treatment C3d-positive DSA was predictive of poor graft survival.				Multivariable Cox proportional hazard model. Pre-treatment C3d-positive DSA was predictive of poor graft survival.			
Variable	Hazard ratio	95% CI	p-value	Variable	Hazard ratio	95% CI	p-value	Variable	Hazard ratio	95% CI	p-value
Age	1	0.97 –1.03	0.84	Age	1	0.97-1.04	0.78	Age	1.006	0.97 – 1.04	0.737
Gender, female	1.88	0.87 –4.11	0.11	Gender, female	1.97	0.91-4.27	0.09	Gender, female	1.909	0.887-4.11	0.098
Previous transplants	7.03	2.24-22.12	*0.001*	Previous transplants	7.15	2.24-22.80	*0.001*	Previous transplants	7.335	2.30 – 23.32	*0.001*
ESRD duration	0.93	0.89-0.98	*0.014*	ESRD duration	0.94	0.89-0.98	*0.015*	ESRD duration	0.935	0.89 – 0.99	*0.014*
Total mismatch	0.94	0.73-1.23	0.66	Total mismatch	0.96	0.74-1.25	0.77	Total mismatch	0.953	0.73 – 1.24	0.719
Early AMR	1.36	0.68 -	0.38	Early AMR	1.31	0.65-2.62	0.45	Early AMR	1.389	0.69 – 2.79	0.355
IgG DSA highest MFI	1	0.99 – 1.00	0.69	IgG DSA MFI ≥8000	1.56	0.69-3.50	0.284	IgG DSA MFI ≥5000	1.288	0.51 – 3.23	0.589
Pre-treatment C3d positive DSA	3.29	1.37-7.86	*0.007*	Pre-treatment C3d positive DSA	2.98	1.29-6.88	*0.011*	Pre-treatment C3d positive DSA	3.319	1.45 - 7.58	*0.004*

Table 3: In multivariable Cox proportional hazard model, pre-treatment and day 14 C3d-positive DSAs were associated with poor graft survival

Variable	Hazard ratio	95% CI	*p*-value
Age	0.80	0.09-6.80	0.840
Gender, female	1.45	0.55-3.81	0.45
Previous transplants	4.55	1.19-17.41	*0.027*
ESRD duration	0.09	0.01-0.63	*0.016*
Total mismatch	0.16	0.01-2.23	0.18
Early AMR	0.91	0.39-2.12	0.83
IgG DSA highest MFI	2.6	0.41-16.65	0.31
Both Pre-treatment and day14 C3d+DSA	4.56	1.46-14.40	*0.009*

Figure Legends

Figure 1: Adjusted Survival analysis of entire cohort with IgG DSA MFI as continuous variable (Model 1). Death censored five-year survival probability of graft in C3d-positive DSA group was lower at 59.6% compared to 84% in the C3d-negative DSA group. Hazard ratio of 3.29 (95%CI 1.37 – 7.86).

Figure 2: Adjusted survival analysis of FC crossmatch patients including IgG DSA MFI as continuous variable (Model 1).Death censored five-year survival probability of C3d-positive DSA cases were lower at 64.8% compared to 90.6% in the C3d-negative DSA cases. Hazard ratio of 9.8 (95%CI 1.6, 59.4).

Figure 3: Kaplan Meier analysis: Death censored five-year survival probability of class I C3d-positive DSA cases were marginally lower at 73.3% compared to 75.5% in class I C3d-negative DSA cases. Relative risk of graft failure was 1.06 (95% CI 0.33, 3.42).

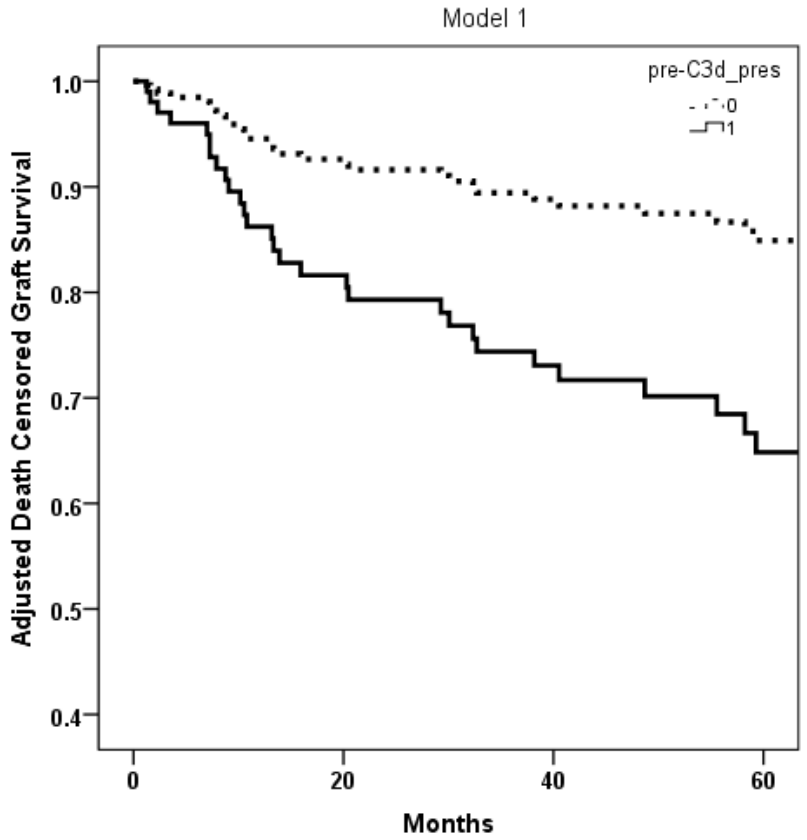
Figure 4: Kaplan Meier analysis: Death censored five-year survival probability of class II C3d-positive DSA cases were lower at 60.7% compared to 80.6% in class II C3d-negative DSA cases. Relative risk of graft failure was 2.02 (95% CI 1.04, 3.94).

Figure 5: Kaplan Meier analysis: Death censored five-year survival probability of class I + II C3d-positive DSA cases were lower at 50% compared to 80.7% in other cases. Relative risk of graft failure was 2.05 (95% CI 0.73, 5.78).

Figure 6: Relative risks of death censored five-year graft failure as per C3d-DSA status at pre-treatment and day 14 compared to cases with pre-treatment and day 14 C3d-negative DSAs group

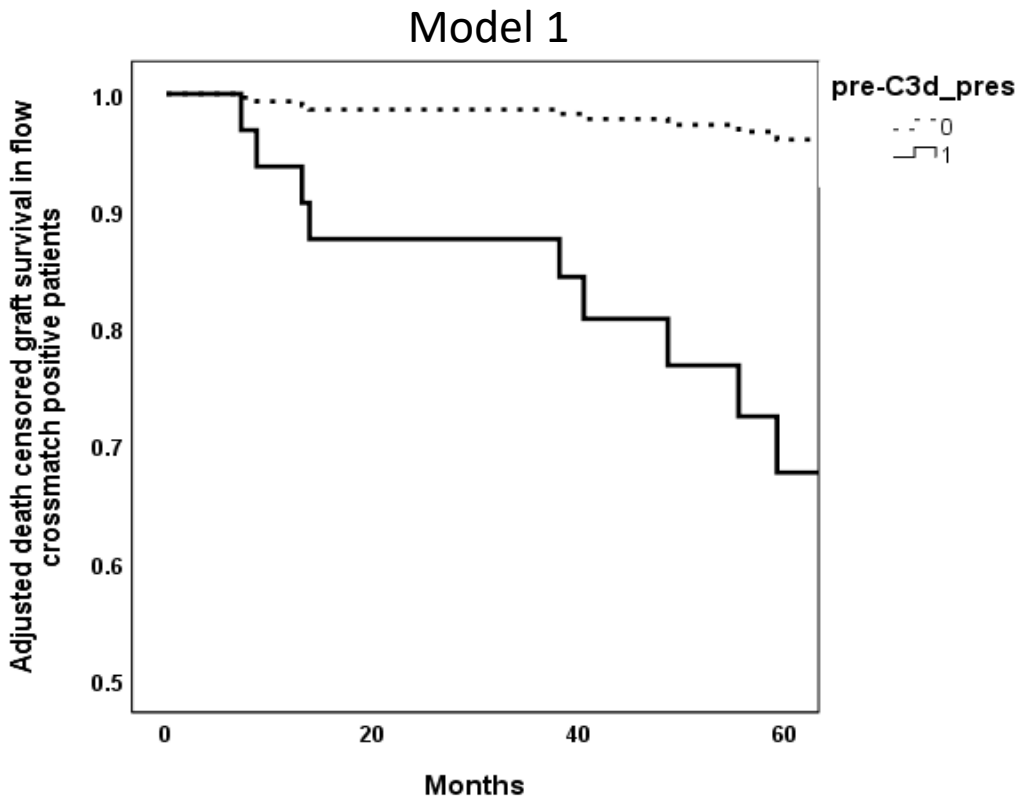
Figure 7: Adjusted Survival analysis including IgG DSA MFI as continuous variable (Model 1). Death censored five-year survival probability of graft in pre-treatment and day 14 C3d-positive DSA group was lower at 43.5% compared to 87.2% in the C3d-negative DSA group. Hazard ratio of 4.56 (95%CI 1.46, 14.39).

Table of contents for supplemental file



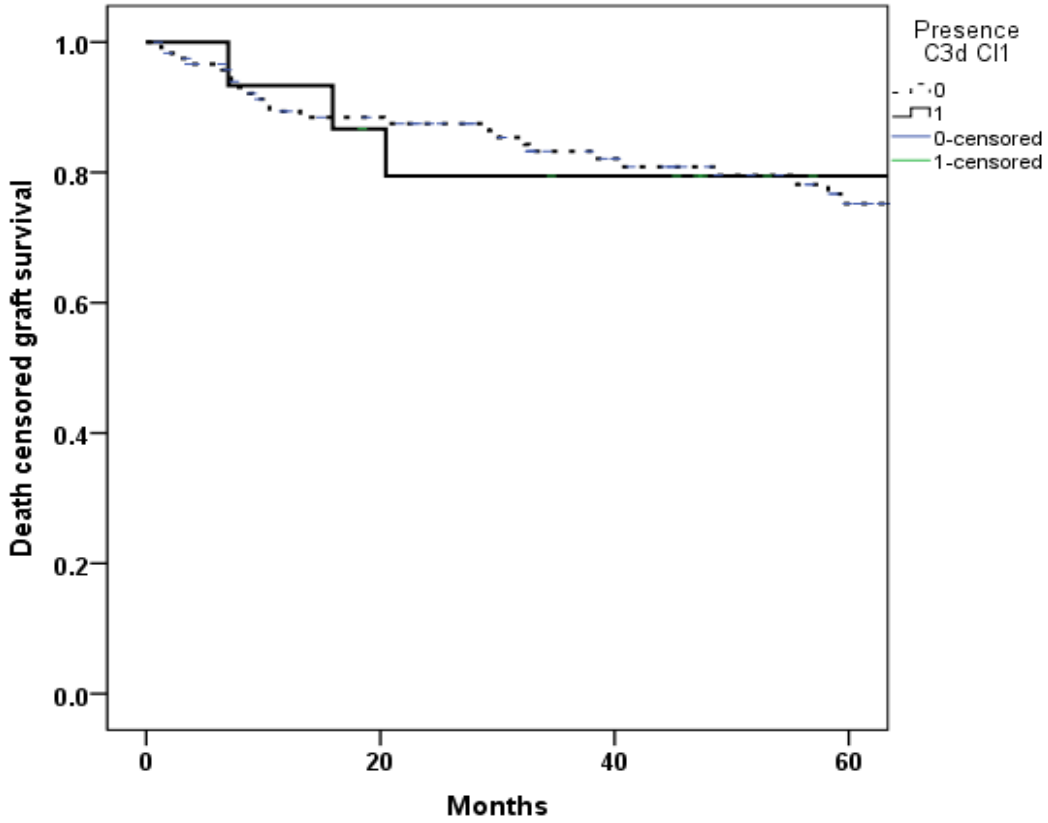
At risk	0	20	40	60
Pre C3d neg DSA	87	69	55	39
Pre C3d pos DSA	52	37	26	16

Figure 1: Adjusted Survival analysis of entire cohort with IgG DSA MFI as continuous variable (Model 1). Death censored five-year survival probability of graft in C3d-positive DSA group was lower at 59.6% compared to 84% in the C3d-negative DSA group. Hazard ratio of 3.29 (95%CI 1.37 – 7.86).



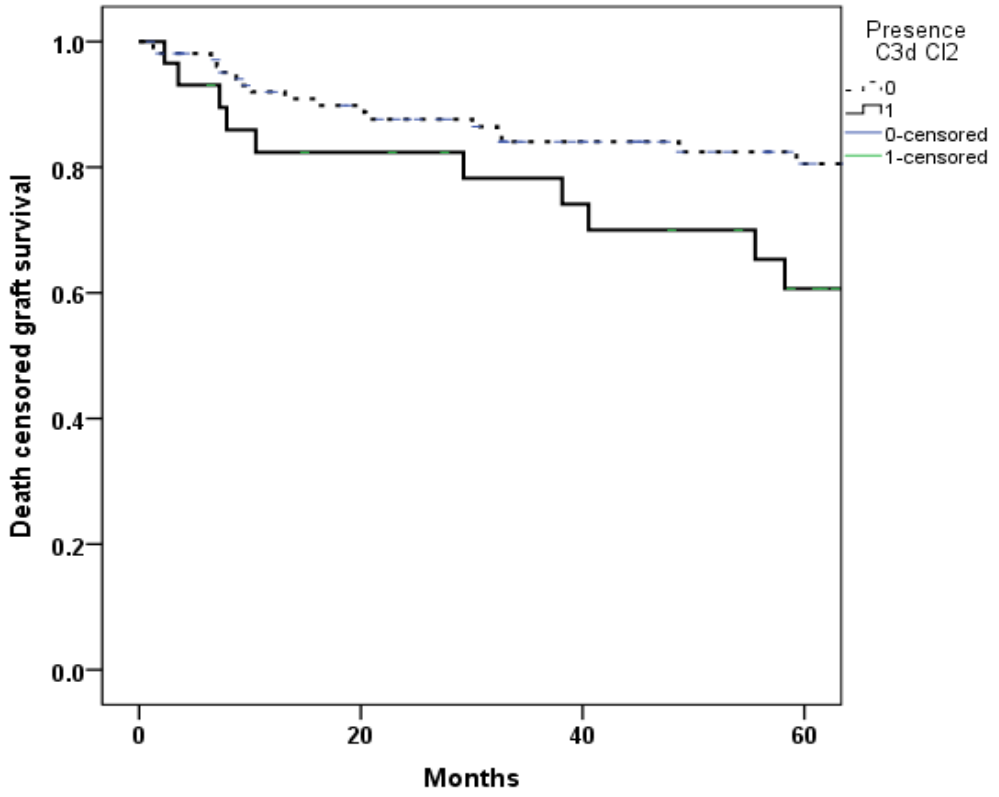
At risk	0	20	40	60
Pre C3d neg DSA	56	48	41	30
Pre C3d pos DSA	17	14	11	6

Figure 2: Adjusted survival analysis of FC crossmatch patients including IgG DSA MFI as continuous variable (Model 1). Death censored five-year survival probability of C3d-positive DSA cases were lower at 64.8% compared to 90.6% in the C3d-negative DSA cases. Hazard ratio of 9.8 (95%CI 1.6, 59.4).



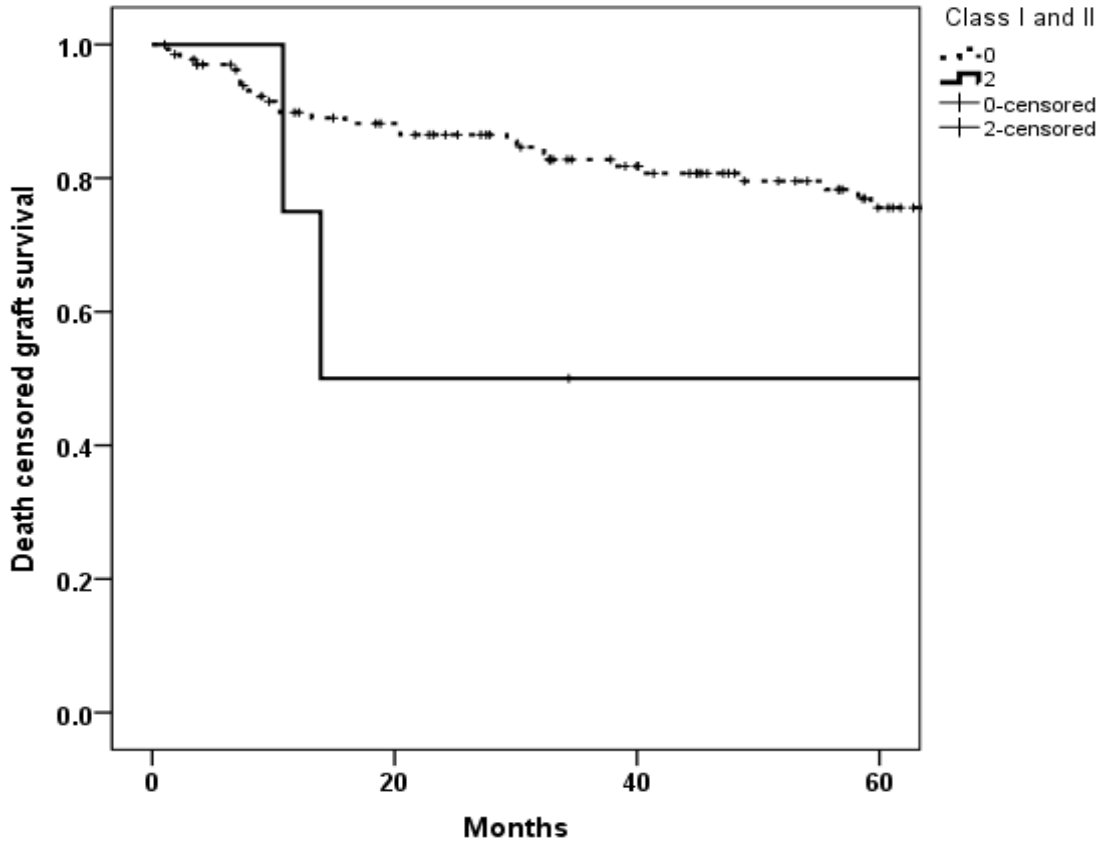
At risk	0	20	40	60
Pre-treatment class I C3d-neg DSA	120	100	80	60
Pre-treatment class I C3d pos DSA only	15	0	0	0

Figure 3: Kaplan Meier analysis: Death censored five-year survival probability of class I C3d-positive DSA cases were marginally lower at 73.3% compared to 75.5% in C3d-negative DSA and class II C3d-positive DSA cases. Relative risk of graft failure was 1.06 (95% CI 0.33, 3.42).



At risk	0	20	40	60
Pre-treatment class II C3d neg DSA	106	86	66	46
Pre-treatment class II C3d pos DSA only	29	9	0	0

Figure 4: Kaplan Meier analysis: Death censored five-year survival probability of class II C3d-positive DSA cases were lower at 60.7% compared to 80.6% in C3d-negative DSA and class I C3d-positive DSA cases.
Relative risk of graft failure was 2.02 (95% CI 1.04, 3.94).



At risk	0	20	40	60
Pre-treatment class I +II C3d neg DSA	135	115	95	75
Pre-treatment class I + II C3d pos DSA only	4	0	0	0

Figure 5: Kaplan Meier analysis: Death censored five-year survival probability of class I + II C3d-positive DSA cases were lower at 50% compared to 80.7% in class I and class II and C3d-negative DSA cases. Relative risk of graft failure was 2.05 (95% CI 0.73, 5.78).

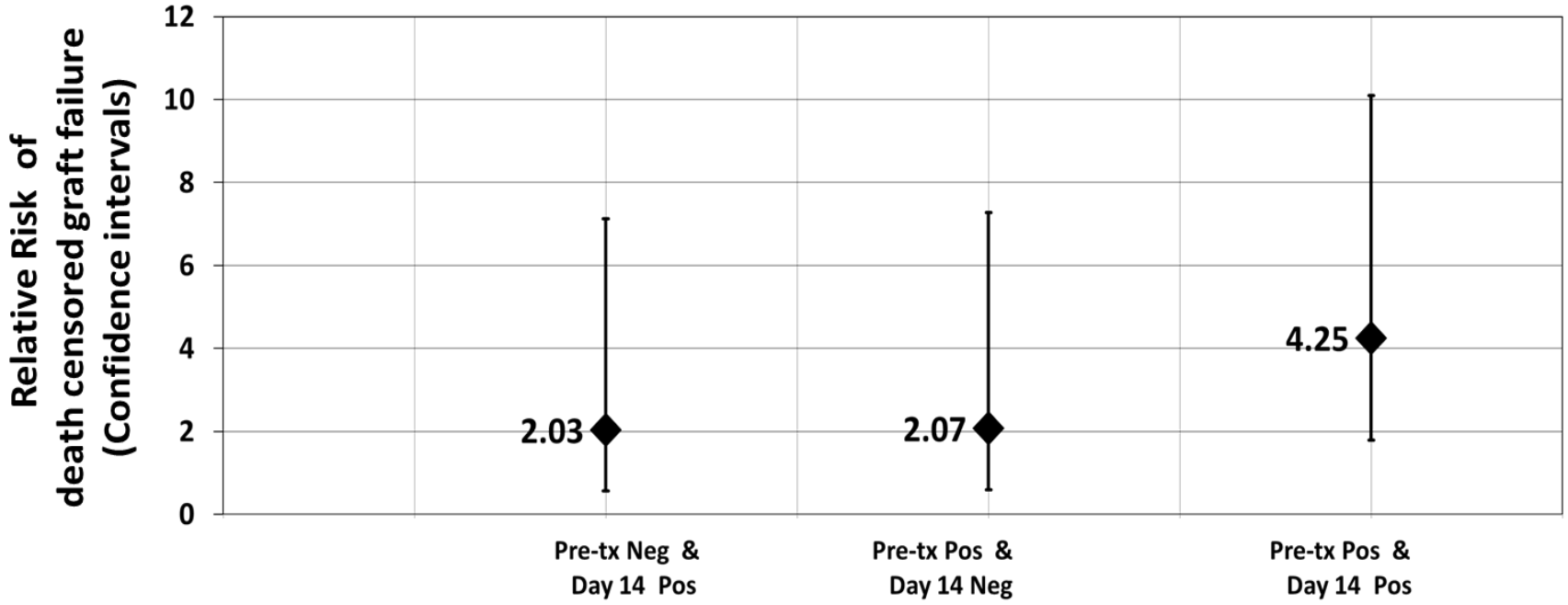
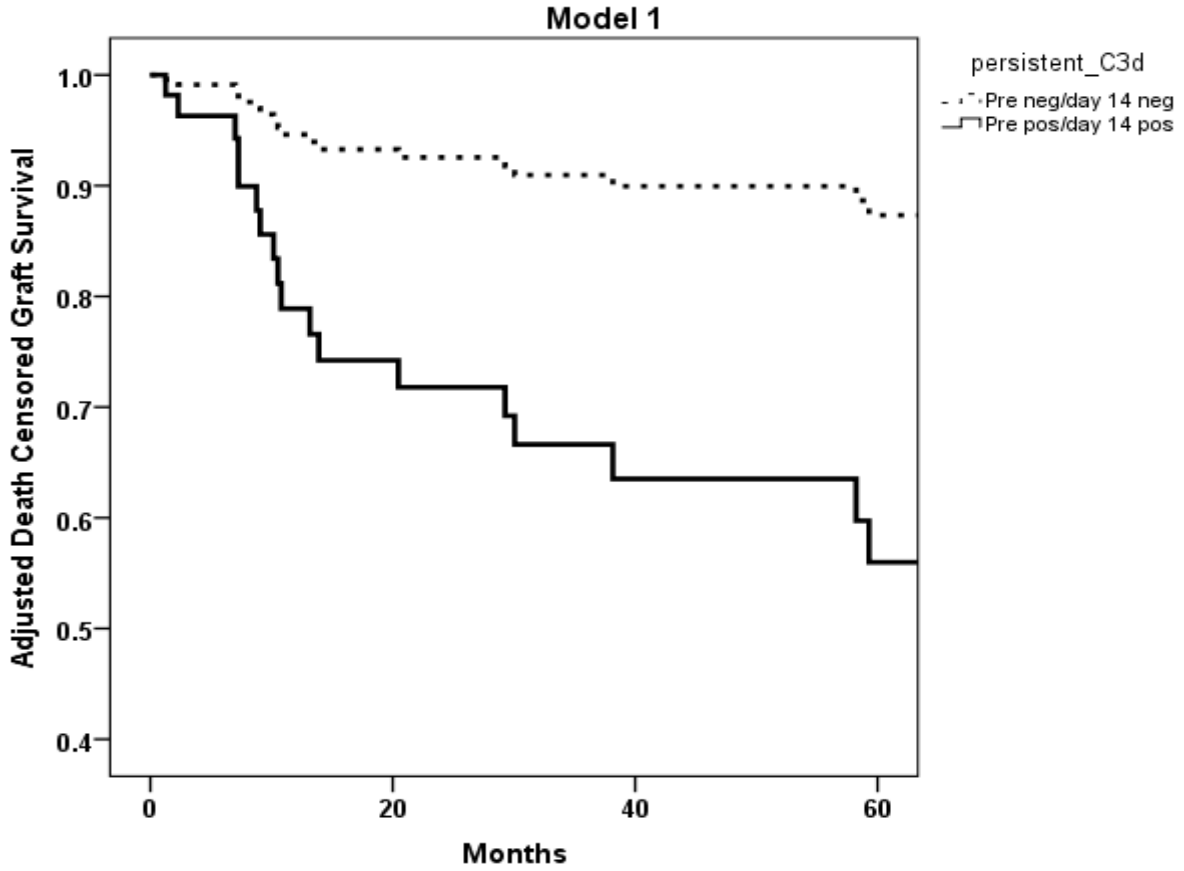


Figure 6: Relative risks of death censored five-year graft failure as per C3d-DSA status at pre-treatment and day 14 compared to cases with pre-treatment and day 14 C3d-negative DSA group



At risk	0	20	40	60
Pre C3d neg /day 14 C3d neg DSA	57	45	37	28
Pre C3d -pos/day 14-pos DSA	24	16	10	7

Figure 7: Adjusted Survival analysis including IgG DSA MFI as continuous variable (Model 1). Death censored five-year survival probability of graft in pre-treatment and day 14 C3d-positive DSA group was lower at 43.5% compared to 87.2% in the C3d-negative DSA group. Hazard ratio of 4.56 (95%CI 1.46, 14.39).